Impaired mitochondrial activity explains platelet dysfunction in thrombocytopenic cancer patients undergoing chemotherapy

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Online data supplement

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Supplemental Methods

Materials

Apyrase, bovine serum albumin (BSA), calcium chloride, the citrate synthase activity kit, dimethylsulfoxide, glucose, magnesium chloride and thrombin were obtained from Sigma (St. Louis, MO, USA). Collagen related peptide (CRP) was purchased from Dr. R. Farndale (Cambridge, UK). ABT-737, 2MeS-ADP and thapsigargin were from SantaCruz Biotechnology (Santa Cruz, CA, USA); fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody against active integrin $\alpha_{IIb}\beta_3$ and FITC-conjugated anti-CD61 antibody from Becton-Dickinson Bioscience (Franklin Lakes, NJ, USA). Alexa Fluor 546 conjugated human fibrinogen; Fluo-4 AM; H_2DCFDA and NuPAGE LDS sample buffer NOVEX were from Invitrogen Life Technologies (Bleiswijk, The Netherlands). FITC-conjugated anti-P-selectin antibody came from Beckman Coulter, rabbit anti-α-tubulin antibody from Abcam (Cambridge, UK) and annexin-A5 FITC-conjugated was from PharmaTarget (Maastricht, The Netherlands). FITC-conjugated anti-GPIbα antibody was from Sanquin (Amsterdam, the Netherlands) and the PE-conjugated anti-GPVI antibody was from Biocytex (Marseille, France). Collagen type I came from Nycomed Pharma (Munich, Germany). Tetramethyl rhodamine methyl ester (TMRE) was from Anaspec (San Jose, CA, USA); Q-VD-Oph from Calbiochem (San Diego, CA, USA) and the fluorometric caspase-3 activity assay from R&D Systems (Minneapolis, MN, USA). Prestained SDS-PAGE standards were from BioRad (Hercules, CA, USA) and SuperSignal West pico chemiluminescent substrate came from Thermo Scientific (Waltham, MA, USA). Actin FSL Activated PTT reagent, Thrombin reagent and Innovance D-dimer test were from Siemens Healthcare Diagnostics (Den Haag, The Netherlands). Antibody against kindlin-3 was purchased at Cell Signaling Technology (Danvers, MA, USA).

Preparation of washed platelets and plasma

Platelet isolation procedures were adapted for measurements with low platelet numbers (median 0.36×10^8 platelets per blood sample). Platelet-rich plasma (PRP) was obtained from citrated blood by centrifugation at 290 g for 2 min. For preparation of washed platelets, PRP in eppendorf tubes was supplemented with 1:10 v/v acidic citrate dextrose (ACD, 80 mM trisodium citrate, 52 mM citric acid and 180 mM glucose) and centrifuged at 2230 g for 2 min.[1] Pelleted platelets were resuspended in Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% bovine serum albumin (BSA). After another centrifugation step (2230 g for 2 min) in the presence of 1:15 ACD + 1 U/mL apyrase, pelleted platelets were resuspended in Hepes buffer pH 7.45 (10

mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose and 0.1% BSA). Platelet count was determined with a Sysmex XP300/XN9000 thrombocounter (Kobe, Japan). Platelet-free plasma was prepared, as described before.[1]

Whole blood cell count and coagulation tests

Whole blood cell count in K_2-EDTA anticoagulated blood was determined using a Sysmex XN-9000 analyzer. Platelet-free plasma was analyzed using a Sysmex CS-2100i analyzer and Dade reagents for: activated partial thromboplastin time (aPTT, Actin FSL), prothrombin time (PT, Innovin), thrombin time (Thrombin reagent), fibrinogen, D-dimers (Innovance) and von Willebrand factor antigen. Coagulation factor activities were determined, as described previously.[2, 3] Normal pooled plasma from healthy controls was used as a reference.

Flow cytometric analysis of platelet activation

To measure platelet activation processes, washed platelets from patients and control subjects were normalized to 10×10^6 platelets/mL. We preferred to use platelets from healthy volunteers as controls and adapt the platelet count, since in cases of immune or congenital thrombocytopenia the underlying acquired or genetic alterations in platelets may also affect platelet functions. Platelets were activated with thrombin (4 nM), CRP-XL (10 µg/mL) or 2MeS-ADP (1 µM) in the presence of 2 mM $CaCl_2$ for 15 min. Integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression were assessed, following labeling with FITC-conjugated PAC-1 antibody (1.25 µg/mL) or FITC-conjugated anti-P-selectin antibody (5 µg/mL), as described before.[4] Receptor expression was determined by post-labeling of platelets with anti-CD61 FITC conjugated antibody (1:10), anti-GPIbα FITC conjugated antibody (1:20) or anti-GPVI PE conjugated antibody (1:20). For detection of apoptotic phosphatidylserine (PS) exposure, washed platelets (10×10^6/mL) were stimulated with ABT-737 (5-10 µM) for 150 min.[5] Sub-samples were stained with FITC-annexin A5 (0.25 µg/mL) in the presence of 2 mM $CaCl_2$. Platelets were pretreated with inhibitor or vehicle for 15 min, where indicated. Samples were analyzed using a BD Accuri C6 flow cytometer and software (Becton-Dickinson Bioscience, Franklin Lakes, NJ, USA).[5]

To assess intracellular Ca^{2+} rises, washed platelets were loaded with the Ca^{2+} probe Fluo-4 AM (8 µM) in the presence of Pluronic F-127 (0.4 µg/mL) for 45 min, as described.[5] After removal of excess probe by a wash step, Fluo-4-loaded platelets were resuspended into Hepes buffer pH 7.45 (2×10^6/mL) supplemented with 2 mM $CaCl_2$, and then activated with thrombin (4 nM), CRP-XL (10 µg/mL) or the SERCA pump inhibitor thapsigargin (0.5

µM). Fluorescence changes were monitored in time, and expressed as increases over basal fluorescence, indicative of rises in cytosolic Ca^{2+} concentrations.[5]

Platelet spreading on fibrinogen

Washed glass coverslips were coated with fibrinogen (100 µg/mL) and blocked with 1% BSA. Washed platelets (10 x 10^6/mL) were allowed to adhere and spread for 10 min,[6] after which brightfield images were captured using a Zeiss LSM7 confocal line-scanning microscope (Oberkochen, Germany) equipped with a 63x oil immersion objective.[7] Spreading state per platelet was classified in three stages based on morphology: *(i)* filopodia, *(ii)* lamellipodia, or *(iii)* fully spread. The analysis was executed blinded.

Mitochondrial function and morphology

To detect mitochondrial depolarization, washed platelets from patients and controls (10 x 10^6/mL) were loaded with the mitochondrial membrane potential dye, tetramethylrhodamine, ethyl ester (TMRE, 50 nM) for 30 min at room temperature. Using flow cytometry, mean fluorescence intensities of TMRE were assessed to evaluate the mitochondrial membrane potential.[5]

Mitochondrial respiration was determined by high-resolution respirometry with an Oroboros Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria), at 37º C and room-air saturated oxygen tension, according to previously established methodology.[8] Briefly, washed platelets were resuspended in buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH_2PO_4, 1 mM EGTA, 5 mM $MgCl_2$, 5 mg/mL BSA, 5 µM pyruvate and 2 µM malate, pH 7.1) and allowed to stabilize on ice for 30 min. Washed platelets (final concentration: 50 x 10^6/mL) were added to the respiration chamber, which contained buffer Z, supplemented with 5 mM pyruvate, 0.5 mM malate and 50 µg/mL saponin. After approximately 15 min, when stable respiration was reached, the OXPHOS substrates ADP (5 mM), glutamate (10 mM), succinate (10 mM) and cytochrome c (5 mM) were sequentially added in 10 min increments. Oxygen concentration and oxygen flux (the negative time derivative of oxygen consumption) were monitored in real time.

As an independent read-out of mitochondrial content, citrate synthase activity was determined in lysates of washed platelets (5 x 10^6), using a colorimetric assay (Sigma, St. Louis, MO, USA). Enzymatic activity was determined at 37° C from absorbance changes at 412 nm, according to the manufacturer's protocol.

For transmission electron microscopy, washed platelets in the presence of 10 nM iloprost were fixed during 1 h with 1.5% glutaraldehyde in phosphate-buffered saline. Samples were then embedded in Epon and processed, as described before.[9]

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Reactive oxygen species (ROS)

To detect ROS, platelets (10 x 10^6 platelets/mL) from patients and controls were loaded with H$_2$DCFDA (10 µM) for 30 minutes at 37°C. Subsequently, subsamples were taken and fluorescence was measured using flow cytometry. Platelets of healthy controls were stimulated with 100 µM CCCP as a positive control.

Western blot analysis

Washed platelets in Hepes buffer 7.45 were lysed (1:4) into 4x ice-cold lysis buffer (600 mM NaCl, 40 mM Tris, 4 mM EGTA, 4 mM EDTA, 4% Nonidet-P40, pH 7.5). Protein content was quantified using a micro BCA protein assay (Thermo Scientific, Waltham, MA, USA). Samples were denatured by adding 1:4 NuPAGE LDS sample buffer, and subsequently heated at 95 °C for 5 min. The denatured samples were separated on an 8% SDS-polyacrylamide gel, and then transferred to a PVDF blotting membrane. Immunoblotting was performed with antibodies against kindlin-3 (1:1000) or α-tubulin (1:1000; loading control) overnight at 4°C, followed by staining using a horseradish peroxidase (HRP) coupled secondary antibody for 1 h at room temperature. After incubation with SuperSignal West pico-chemiluminescent substrate, protein bands were visualized using an ImageQuant LAS-4000 mini system (Wauwatosa, WI, USA).

Caspase activity

Caspase activity was assessed in washed platelets (10 x 10^6/mL) treated with vehicle or ABT-737 (10 µM) for 90 min at 37° C, as described before.[5]

Supplemental Results

Table S1: Coagulation parameters and factor activities of patient blood samples. Factor activities were assayed relatively to Standard Human Plasma (SHP) and are expressed as % activity. Samples without prior platelet transfusion. (Medians + interquartile ranges)

Coagulation parameters	Patient cohort	Reference range
Fibrinogen (g/L)	4.2 (3.4-5.1)	1.7-4.0
d-Dimer (ng/mL)	2166 (1395-4276)	< 500
aPTT (s)	29.2 (26.5-33.4)	23.0-32.0
PT (s)	11.0 (10.6-11.6)	9.9-11.5
Thrombin time (s)	15.8 (14.9-16.7)	15.0-22.0
vWF (%)	251 (200-306)	40-190
Prothrombin (%)	92 (85-100)	70-130
Factor V (%)	126 (101-150)	70-130
Factor VII (%)	58 (47-73)*	70-130
Factor VIII (%)	172 (144-197)	50-200
Factor IX (%)	127 (114-139)	60-140
Factor X (%)	77 (66-84)	60-140
Factor XI (%)	106 (85-120)	60-140
Factor XII (%)	93 (69-104)	60-140
Factor XIII (%)	105 (90-129)	70-140

Table S2: Chemotherapeutic drugs taken by included patients, classified according to the main action mechanism. Drug classes: A, antitumor antibiotics & topo-isomerase II inhibitors; B, antimetabolites; C, alkylating agents; D, mitotic inhibitors; E, other.

Class	Drug
A	daunorubicin, doxorubicin, etoposide, mitoxantrone, idarubicin
B	cytarabine (ara-C), fludarabine
C	busulfan, carmustine (BCNU), cyclofosfamide, melphalan
D	vincristin
E	lenalidomide, tosedostat, retuximab

Table S3: Numbers of included patients taking chemotherapeutic drugs of different classes, alone or in combination. For the list of individual drugs, see Table S1.

Diagnosis	Drug class				
	A anti-tumor & TI inhibitor	B anti-metabolite	C alkylating agent	D mitotic inhibitor	E other
AML/ALL, *n*	24	29	11	1	10
Lymphoma, *n*	13	15	15	1	1
Multiple myeloma, *n*	0	0	21	0	0
Other, *n*	0	1	3	0	0
Total, *n* (%)	37 (25.5%)	45 (31.0%)	50 (34.5%)	2 (1.4%)	11 (7.6%)

Table S4: Hematologic parameters of blood samples from patients before and 1 h after platelet transfusion. Data are expressed as medians (interquartile ranges)

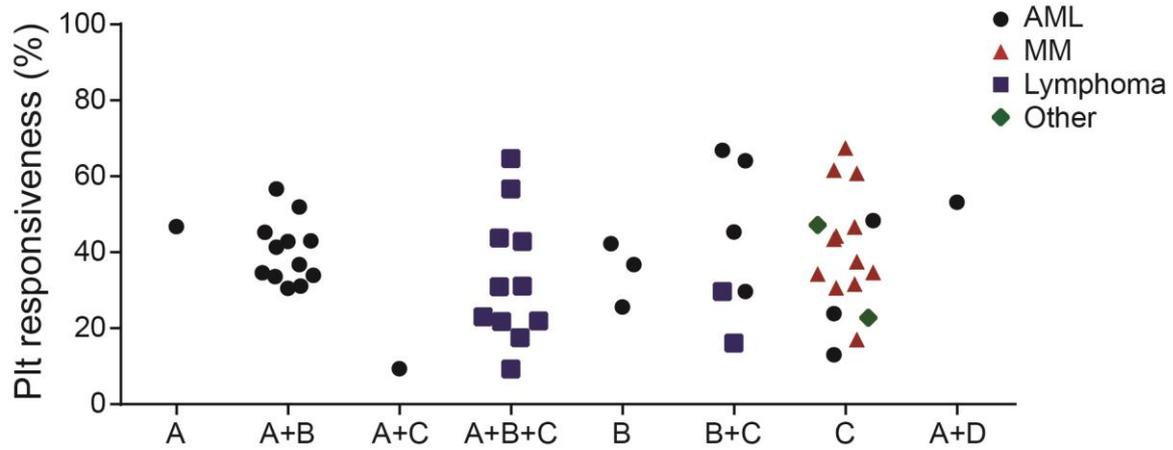| Parameter	Before transfusion	After transfusion
Leukocyte count (x 10^9/L)	0.14 (0.04-0.41)	0.12 (0.04-0.44)
Hemoglobin (mM)	5.6 (5.1-6.2)	5.2 (4.7-5.7)
Platelet count (x 10^9/L)	6 (4-8)	33 (24.3-43.8)
Absolute immature platelet number (x 10^9/L)	0.21 (0.07-0.38)	1.17 (0.69-1.60)
Immature platelet fraction (%)	3.8 (2.2-6.3)	3.6 (2.7-4.2)
Corrected count increment	*n.a.*	14.8 (11.3-18.0)

Figure S1: Variable impairment in platelet responsiveness is independent of chemotherapy treatment regimen of patients. Integrin activation and P-selectin expression of washed platelets from patients was determined, as for Figure 1. Overall platelet responsiveness was defined per patient as the mean of percentages of platelets positive for integrin activation and P-selectin after stimulation with thrombin, CRP-XL or ADP. Patients were grouped based on treatment with chemotherapeutics into one of more classes (see Table S2): A, antitumor antibiotics & topo-isomerase inhibitors; B, antimetabolites; C, alkylating agents; D, alkaloids. Data from 52 patients (AML: *n*=25, multiple myeloma: *n*=12, lymphoma: *n*=13, other: *n*=2).
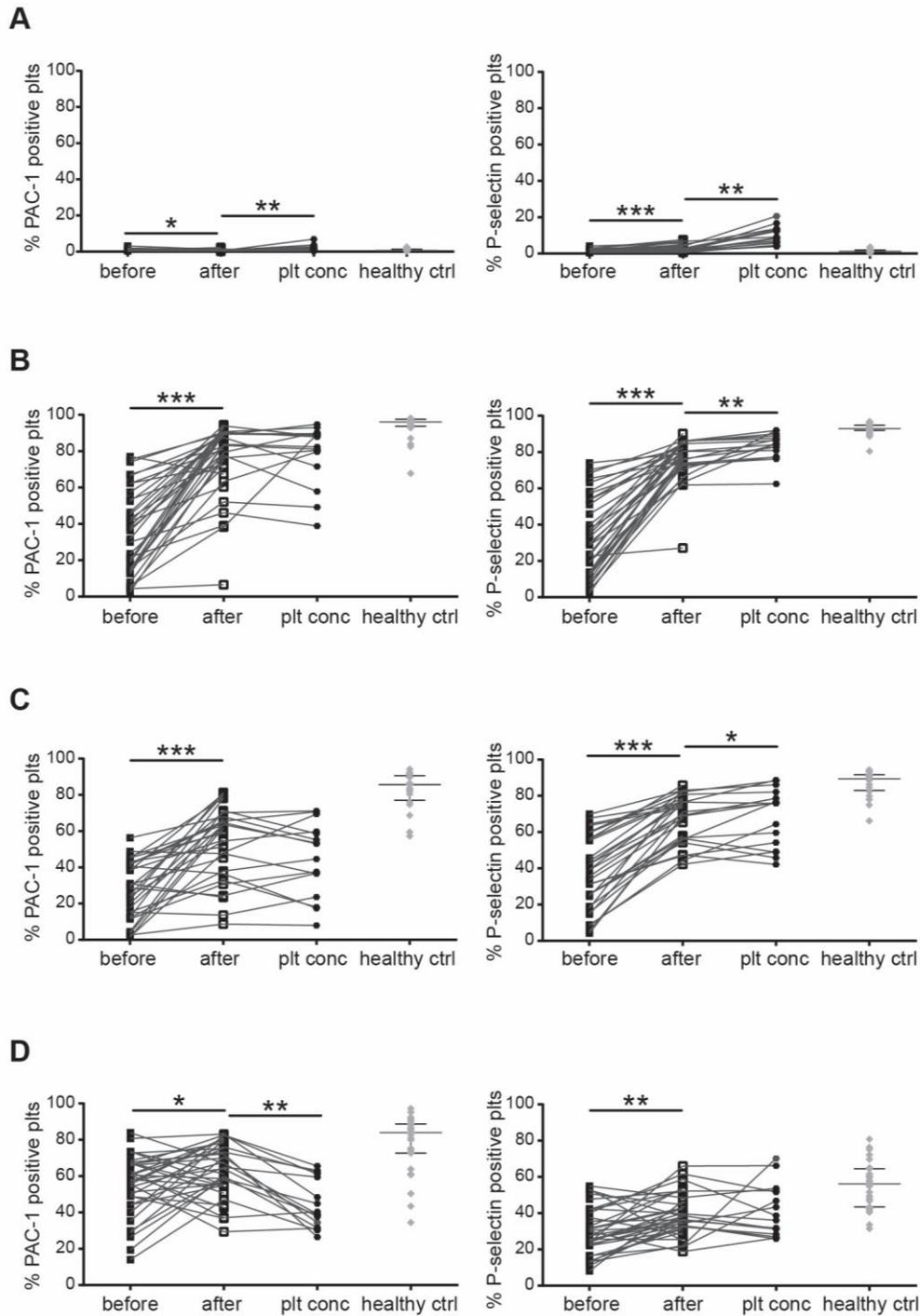
Figure S2: Transfusion partly normalizes platelet responsiveness in chemotherapy treated patients with thrombocytopenia

Washed platelets (10×10^6/mL) analyzed from healthy controls, patients with chemotherapy-induced thrombocytopenia before and after transfusion, and from the transfused platelet concentrates. Integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression were measured in resting platelets (**A**), and after stimulation with thrombin (**B**), CRP-XL (**C**) or 2MeS-ADP (**D**). Median values with IQR for patients (n=36: treatment classes: A+B: n=7; A+B+C: n=10; B+C: n=4; C: n=11) and platelet concentrates (n=16) and control subjects (n=27); *$p < 0.05$, **$p<0.01$ and ***$p<0.001$.
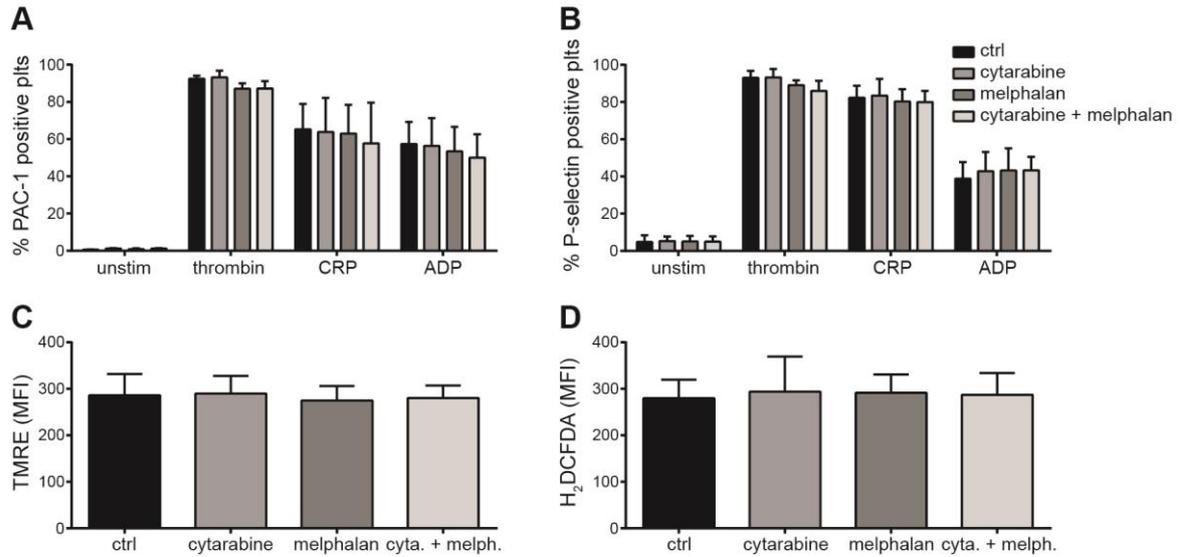
Figure S3: *Ex vivo* treatment with chemotherapeutic agents does not influence platelet functionality. Whole blood from healthy control subjects was incubated with clinically relevant concentrations of cytarabine (70 µg/mL) and/or melphalan (50 µg/mL) for 60 min at 37° C. Subsequently, platelets were isolated, and platelet reactivity (Integrin $\alpha_{IIb}\beta_3$ (**A**) and P-selectin expression (**B**)), mitochondrial membrane potential (TMRE) (**C**), and ROS (H$_2$DCFDA) (**D**) were measured. Results are depicted as means plus SD, n=4, *p<0.05.

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